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Definition of three minimal T helper cell epitopes of rubella virus E1 glycoprotein

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SUMMARY

To characterize T cell-recognized epitopes on rubella virus (RV) E1 glycoprotein, IL-2-dependent RV-specific T cell lines were established from 14 rubella-seropositive healthy donors. The responses of these lines were studied by using a panel of 94 partially overlapping synthetic peptides of 15 amino acids (aa) length covering the known nucleotide sequence of RVE1 glycoprotein. Two to seven peptide-defined epitopes were recognized by the T cell lines, but a large interindividual variation was found. T cell reactivity was most often localized to the regions between aa 276 and 290, aa 381 and 395 and aa 410 and 420. Analysis of overlapping, truncated peptides revealed three minimal T helper cell epitopes VIGSQARK, KFVTAALLN and RVIDPAAQ in aa positions 280–287, 385–393 and 412–419, respectively.

Keywords rubella virus T cell epitopes E1 glycoprotein

INTRODUCTION

Rubella is a mild disease of childhood which is now rare in developed countries because of wide scale immunization [1]. Infection of the fetus during the first trimester with rubella virus leads to the congenital rubella syndrome with associated organ damage. Before widespread vaccination, rubella was among the most important causes of congenital heart disease, deafness, blindness, mental retardation and other malformations, and is also associated with an increased risk of autoimmune diabetes [1]. Adults, especially female subjects, may develop transient arthritis during natural infection or after receiving the attenuated live vaccine, and it has been suggested that the virus is involved in some forms of juvenile and rheumatoid arthritis [2]. The live attenuated vaccine virus causes a mild infection resembling the natural one, and might in principle as well be involved in induction of autoimmune responses. That is one reason why rubella has been brought up as a candidate for development of subunit vaccines.

Rubella virus (RV) is an enveloped positive strand RNA virus of the Togaviridae family [1]. It contains three major structural proteins: glycoproteins E1 and E2, and an internal capsid protein C. The E1 glycoprotein bears most of the neutralization sites [3,4], and seems to be the major target of cell-mediated immune responses [5]. $CD4^+$ T lymphocytes initiate and provide help for both the cell-mediated and humoral immune responses. T helper cells can not bind soluble antigen but recognize a processed peptide fragment in association with class II MHC molecules on the surface of antigen-presenting cells. In general, epitopes recognized by T cells are short peptides bound to a specific groove in the MHC molecule. The rules of this binding are just taking shape [6–9].

In the present study we used IL-2-dependent RV-specific T cell lines and panels of E1-specific synthetic peptides to identify and characterize epitopes able to initiate specific immune response by activating T helper cells.

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MATERIALS AND METHODS

Rubella virus and synthetic peptides

To prepare the rubella virus (Theiren strain) antigen, VERO cells were infected with low multiplicity of infection and harvested when the cytopathic effect was complete. The cells were scraped into PBS and washed three times with PBS. After disruption of cells by five cycles of freezing and thawing, the debris was removed by centrifugation at 2000 ***g*** for 30 min. The supernatant was then centrifuged at 80 000 ***g*** for 30 min and resuspended in PBS. Virus was inactivated by β-propiolactone at a final concentration of 0·02% at 4°C for 18 h. Ninety-four 15 amino acid (aa) long synthetic peptides with 10 aa overlaps covered the published amino acid sequence of E1 glycoprotein [10]. Peptides were synthesized on polyethylene pins by Fmoc-chemistry and cleaved into buffer solution at pH 7·0 according to Reece *et al.* [11] (Cleavable peptides kit; Chiron Mimotipes Ltd, Clayton, Australia). A final concentration of 1 μg/ml was used in lymphocyte cultures. Three series of truncated 8–15 aa long peptides corresponding to the sequences of peptides 56, 77/78 and 82/83 were synthesized to reveal the structure of the major T cell epitopes.

T cell lines

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized venous blood of healthy RV-seropositive subjects with a history of past rubella infection by Ficoll–Paque gradient centrifugation. To establish antigen-specific T cell lines, PBMC ($2 \cdot 10^6$/ml) were first incubated for 7 days with the RV antigen (2·5 μg/ml) in RPMI 1640 medium supplemented with 7·5% AB serum, gentamycin, HEPES and glutamin. Fresh medium supplemented with IL-2 (20 U/ml; Boehringer Mannheim, Mannheim, Germany) was added thereafter every second or third day. Washed cells were restimulated for the first time 14 days from the beginning of cultures with the RV antigen in presence of irradiated (30 Gy) fresh autologous PBMC as antigen-presenting cells (APC) ($2 \cdot 10^6$ PBMC/ml). IL-2-containing medium (20 U/ml) was added 2–3 days after antigen stimulation and every second or third day thereafter. Cycles of restimulations were performed at 10-day intervals when cell lines were maintained.

Lymphocyte proliferation assay

Responses of T cell lines were tested a minimum of 7 days after the last addition of feeder cells and antigen. A total of 10^4 T cells were incubated in triplicate wells with $2 \cdot 10^4$ APC and different antigens in 200 μl volumes in 96-well round-bottomed microtitre plates for 2 days. Tritiated thymidine (2 μCi/ml) was added 18 h before harvesting the cell cultures on glassfibre filters using Tomtec 93 Mach III Manual Harvester (Tomtec, Orange, CT). Responses of fresh PBMC were studied using $5 \cdot 10^4$ cells/well and 6 days incubation time. Incorporated radioactivity was measured by a Micro-Beta scintillation counter (Wallac Ltd, Turku, Finland). Stimulation index (SI) was calculated by dividing the median of stimulated triplicate culture wells by the median of control responses. A response was considered positive when the SI was more than 2·5.

HLA typing

HLA typing of blood donors was carried out using panels of commercial antisera (Biotest AG, Dreieich, Germany) in standard microlymphocytotoxicity tests. B cells for HLA class II typing were enriched using immunomagnetic beads [12].

RESULTS

In preliminary experiments we did not see proliferative responses when PBMC were stimulated with 94 synthetic peptides covering the known sequence of E1 glycoprotein. Responses appeared when RV-specific T cell lines were established and responses were enhanced after a cycle of restimulation with antigen containing the whole virion.

RV-specific T cell lines were established from 14 donors, and nine (64%) of these lines responded to some of the tested peptides, whereas five other lines responded only to the RV antigen. The results indicated great individual variation among the nine responsive lines. Figure 1 shows some examples of the responses. Peptides 56, 77/78 and 82/83 were most often recognized as T helper cell epitopes (Table 1). Furthermore, consistency of the individual patterns of the responsiveness could be demonstrated in spite of large interindividual variation. For instance, response to peptide 83 was regularly detected in six different RV-specific T cell lines established from the same subject during a period of 3 years. In most cases the responses against these three above mentioned peptides were strong (SI ≥ 2·5) and often approached the responses obtained by the virion antigen. Besides peptides 56 and 77/78 and 82/83, several others stimulated in single cases.

New series of truncated peptides were synthesized to define minimal epitopes in the three antigenic regions most often stimulating T helper cells. Figure 2 shows that peptides consisting of 8–9 aa were able to stimulate in each case. The minimal amino acids for

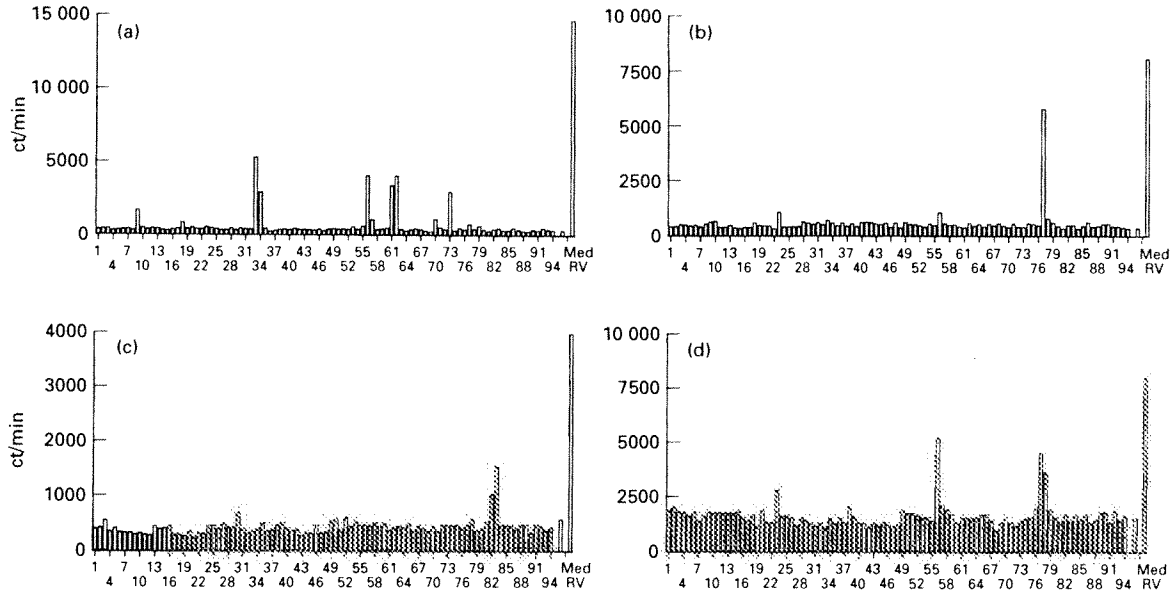

Fig. 1. Four examples of responses to 15 amino acids long peptides overlapping rubella virus E1 glycoprotein by T cell lines from different donors (a–d). Med, Medium; RV, rubella virus; 1–94, various peptides.

Table 1. Responses of rubella virus (RV)-specific human T helper cell lines to 94 overlapping 15 amino acids long peptides derived from the sequence of E1 glycoprotein

T cell lines and HLA-DR type of blood donor	Peptides recognized
1 DR 2,5(12)	77, 78, 82, 83
2 DR 2,4	56, 77, 78
3 DR 3,4	77, 78
4 DR 5(11),8	83
5 DR 5(11),9	9, 18, 33, 34, 56, 57, 61, 62, 70, 73
6 DR 2	23, 56, 77
7 DR 1,4	4, 14, 77
8 DR 4,6(13)	77
9 DR 3,6(13)	16, 56, 63, 75

Only those nine of 14 tested RV lines are included which responded to at least one of the peptides.

peptide 56 were aa 280–287 (VIGSQARK), aa and 385–393 (KFVTAALLN) for peptides 77/78 and for peptides 82/83 aa 412–419 (RVIDPAAQ).

HLA class II typing did not reveal strict associations in the restricted number of T cell lines recognizing each peptide. All of the four DR4$^-$ donors showed a response to peptide 77, but there were also two DR4$^-$ donors (both DR2$^-$) showing a response to this peptide. HLA restriction studies indicated that the peptide could be presented in context of both DR4 and DR2 antigens (data not shown).

DISCUSSION

We identified and further characterized three major T helper cell epitopes in RVE1 glycoprotein. Both fusion proteins and synthetic peptides have been applied to this kind of study [13–16]. Lovett and coworkers detected PBMC responses to a fusion protein covering aa 162–332 in 17 (41%) of 41 studied seropositive donors. None of 18 donors responded to a comparable fusion protein covering aa 344–474 which spans over two of the major epitopes recognized in our study. However, a considerable proportion of donors did not respond to any of the fusion proteins investigated [13].

In preliminary experiments we did not see any responses to synthetic peptides when PBMC were used. Responses appeared only when T cells prestimulated with the RV virion antigen were first enriched as IL-2-dependent lines. Some of the T cell epitopes in influenza virus nucleoprotein have been found by using PBMC, but more epitopes were detectable when restimulated T cell lines were used [17]. There are differences in the strength of proliferation responses obtained with various viral antigens [18], which may reflect number of circulating specific cells. The responses obtained by T cell lines can be considered more specific than those with PBMC, although selection of T cell specificities during *in vitro* stimulation might occur. In our study, the same individual epitopes were, however, consistently found by T cell lines raised at different time points.

Ou and coworkers used 49 overlapping synthetic peptides to cover most of the E1 sequence and T cell lines from different types of seropositive donors [15]. The most commonly found epitope

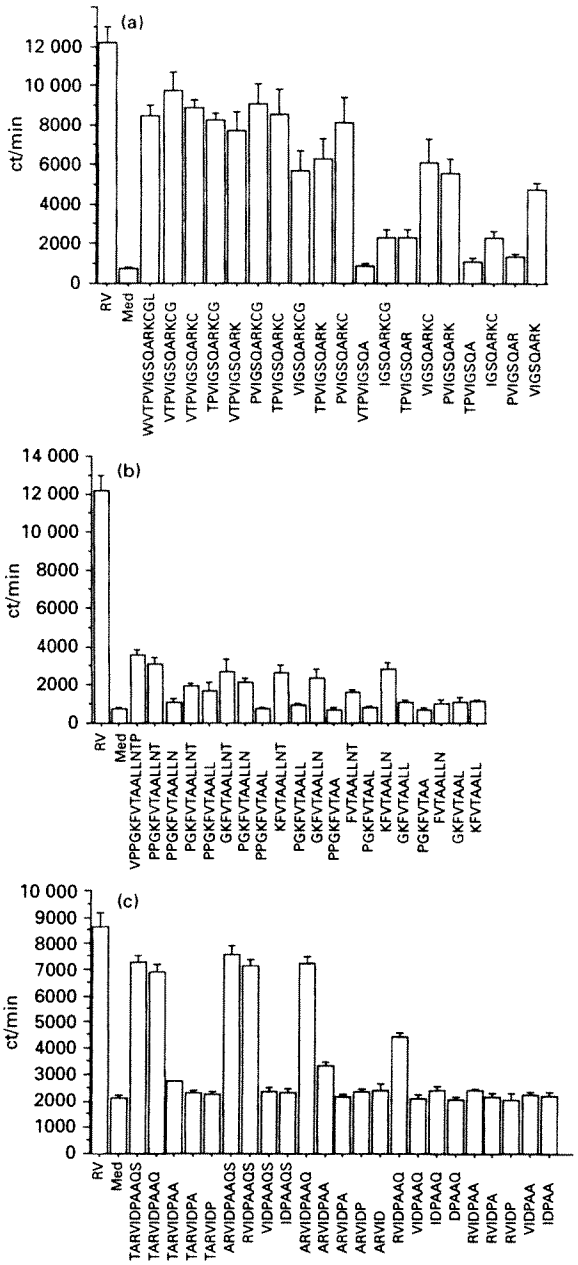

Fig. 2. Search of minimal T cell epitope in three antigenic regions (56, 77, 83) of rubella virus E1 glycoprotein. The responses of T cell lines recognizing each epitope were tested using series of truncated peptides of various lengths. The necessary amino acids from T cell stimulation were aa 280–287 (VIGSQARK) from peptide 56 (a), aa 385–393 (KFVTAALLN) from peptide 77 (b), and aa 412–419 (RVIDPAAQ) from peptide 83 (c).

was detected within residues 358–377 (11/20), but the interindividual variation was great. In our series a corresponding peptide, aa 360–375, was recognized only once. One of our major epitopes, peptide 56 (four of nine responding lines) overlapped a previously

described peptide E1-16 (aa 272–291) which was recognized by five of 20 T cell lines [15]. The two other major epitopes in our series were not included among the peptides studied by Ou and coworkers.

We defined a minimal epitope VIGSQARK (aa 280–287) within the peptide 56.The minimal epitope defined by Ou *et al.* using cytotoxic CD4 clones was 273–282 [19], which partially overlaps with the epitope defined in this study. Clones used by Ou *et al.* were HLA-DR4-restricted and the epitope in this study was defined by a T cell line of a donor with HLA-DR2,4 genotype. Two of the four subjects recognizing the epitope had HLA-DR3 and/or 52 alleles, and this epitope in fact contains the motif i – 3 described by Chicz *et al.* for the peptides restricted by these particular molecules [6]. In this description i means a hydrophobic residue (F, I, L, V or Y) and the third amino acid residue thereafter would be a preferred amino acid (D, N, Q or T) fitting to the pocket in the MHC molecule [6]. Neither of the two other minimal epitopes did strictly fit to the motifs described for any class II molecules, but both had the hydrophobic residue near amino-terminal end found in most MHC bound peptides. In fact, the sets of natural peptides eluted from purified MHC molecules have not completely confirmed strict rules of amino acid position preferences and rigid allele-specific motifs for class II molecule binding [6].

In conclusion, we were able to define some commonly recognized T helper cell epitopes in RVE1 glycoprotein. Our method of using rubella virus antigen to establish the specific T cell lines ensures that the epitopes found by synthetic peptides are naturally processed epitopes. The use of synthetic peptides throughout the procedure may result in non-specific findings; a wide cross-reactivity between microbial peptides has recently been demonstrated at T cell level [20]. Minimal epitopes of nine or even only eight amino acids were able to stimulate T cells. Our results in combination with other recent work demonstrate that no generally recognized T helper cell epitopes exist. However, about half of the individuals recognized one of the epitopes, and peptides restricted for different MHC class II molecules were found to span over the two other major T helper cell epitopes defined in this study. The large interindividual variation of the recognized T helper cell epitopes stresses the need for more than one single component in a candidate peptide vaccine for rubella.

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